PB87-116638

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(U.S.) Environmental Research Lab.-Duluth, MN

Oct 86

U.S. Department of Commerce
National Technical Information Service
NTIS

PB87-116638

EPA/600/D-86/239
October 1986

FUNCTIONAL RESPONSES OF LABORATORY MICROECOSYSTEMS TO CHEMICALS FROM THREE STRUCTURAL GROUPS

by

J. D. Yount
Environmental Research Laboratory
U.S. Environmental Protection Agency
Duluth, MN 55804

L. J. Shannon
Department of Biology
University of Minnesota
Duluth, MN 55812

ENVIRONMENTAL RESEARCH LABORATORY
OFFICE OF RESEARCH AND DEVELOPMENT
U.S. ENVIRONMENTAL PROTECTION AGENCY
DULUTH, MN 55804

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TECHNICAL REPORT DATA *(Please read Instructions on the reverse before completing)*		
1. REPORT NO. EPA/600/D-86/239	2.	3. RECIPIENT'S ACCESSION NO.
4. TITLE AND SUBTITLE Functional Responses of Laboratory Microecosystems to Chemicals from Three Structural Groups.		5. REPORT DATE October 1986 6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) J. David Yount[1] and Lyle J. Shannon[2].		8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS [1]U.S. EPA, Environmental Research Lab-Duluth, 6201 Congdon Boulevard, Duluth, MN 55804; [2]Dept. of Biology, University of Minnesota, Duluth, MN 55812.		10. PROGRAM ELEMENT NO. 11. CONTRACT/GRANT NO.
12. SPONSORING AGENCY NAME AND ADDRESS Environmental Research Laboratory Office of Research and Development U.S. Environmental Protection Agency Duluth, MN 55804		13. TYPE OF REPORT AND PERIOD COVERED 14. SPONSORING AGENCY CODE EPA/600/03
15. SUPPLEMENTARY NOTES		

16. ABSTRACT

Generic mixed-flask culture microecosystems derived from small lake and pond planktonic communities were used to evaluate the effects of selected alcohols, aniline derivatives, and aromatic amides on ecosystem-level state variables. The biogeochemical state of the systems was characterized by pH and dissolved oxygen, which reflect energy flow and nutrient cycling processes within the systems.

Using changes in these variables as indicators of effect, the relative toxicity rankings of compounds within each group were determined and compared with the toxicity rankings established by fathead minnow acute toxicity. The ecosystem-level relative toxicity of three alcohols followed the toxicity rankings based on fathead minnow 96 hr LOEL's (lowest observed effect levels). The toxicity rankings of aniline and three derivatives were similar to those of the alcohols. In both alcohols and anilines, ecosystem-level effect measures became more sensitive than fathead minnow lethality toward the least toxic member of the group. An aromatic amide group of compounds showed the most departure from the fathead minnow toxicity ranking since it included an inhibitor of photosynthesis and an uncoupler of oxidative phosphorylation. The amide compound which was least toxic to fathead minnows was most toxic at the ecosystem level.

These examples can be considered representative of situations that might be encountered in an early stage of ecosystem-level testing. For less well known toxicants, microcosm functional testing could assist in identifying chemicals which require more elaborate test procedures.

KEY WORDS AND DOCUMENT ANALYSIS		
a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field Group
18. DISTRIBUTION STATEMENT Release to public	19. SECURITY CLASS (*This Report*) Unclassified	21. NO. OF PAGES 29
	20. SECURITY CLASS (*This page*) Unclassified	22. PRICE

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DRAFT

Functional responses of laboratory microecosystems to chemicals from three structural groups.

Yount, J. David[1] and Lyle J. Shannon[2]

1. U.S. Environmental Protection Agency, Environmental Research Laboratory, Duluth, MN 55804

2. Department of Biology, University of Minnesota, Duluth, MN 55812

ABSTRACT: Generic mixed-flask culture microecosystems derived from small lake and pond planktonic communities were used to evaluate the effects of selected alcohols, aniline derivatives, and aromatic amides on ecosystem-level state variables. The biogeochemical state of the systems was characterized by pH and dissolved oxygen, which reflect energy flow and nutrient cycling processes within the systems.

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These examples can be considered representative of situations that might be encountered in an early stage of ecosystem-level testing. For less well known toxicants, microcosm functional testing could assist in identifying chemicals which require more elaborate test procedures.

KEY WORDS: microcosms, laboratory microecosystems, toxicity testing, hazard ranking, ecosystem effects, alcohols, anilines, aromatic amides

Introduction

This work was inspired ,in part, by a critical review [1] in which aquatic and terrestrial laboratory multispecies test systems were evaluated for their utility in ecological hazard and risk assessment processes. Since then, numerous authors have addressed the need for methods for environmental effects assessment of chemicals at the community and ecosystem level [2-6]. Cairns [7] has also noted the lack of a systematically generated body of data to determine the usefulness of data generated at one level of biological organization in predicting responses at a higher level of organization. We believe that the work reported here contributes to such a body of data, and that our results provide insight into how ecosystem-level testing might be incorporated at an early stage into a tier-testing scheme.

Leffler [8] has developed a protocol to quantify the effects of chemicals on a few easily measured integrative properties of model ecosystems and to rank chemicals in order of the concentrations required to produce an observable effect. He has hypothesized that these rankings will be consistent among mixed cultures with differing species composition even though the absolute values of the measured variables may not be accurate predictors of specific effect levels in other mixed cultures or in natural ecosystems. Giddings [9] has observed that "the major criterion for such a laboratory system is its ability to generate rankings that are consistent with the actual hazard potential of the chemical in nature, rather than its ability to simulate specific ecosystem effects. Test chemicals could be compared with selected standard reference chemicals to identify those with the greatest potential for

environmental effects.".

In 1983 we began a program to evaluate the utility of Leffler's microcosm testing protocol for routinely testing large numbers of chemicals. We were interested not only in the relative toxicity or hazard rankings produced by the test but also in its sensitivity compared to representative single species tests. We used the extensive fathead minnow (Pimephales promelas) toxicity data base developed at the EPA Environmental Research Laboratory-Duluth (ERLD) to establish a rank order against which relative toxicity to microcosm communities could be compared. This data base was generated in order to develop quantitative structure-activity relationships (QSAR) for toxicants with similar modes of action. From this data base we were therefore able to select three chemical groups containing compounds with different representative modes of action and widely differing toxicity. However, because our primary objective was to examine relative toxicity rankings determined by ecosystem level effects, not to compare the relative sensitivity of ecosystem and single species responses, several of the compounds tested had only fathead minnow toxicity data available.

Detailed analyses of these tests are summarized in other publications [10-12] in which each chemical group is treated separately. The purpose of this paper is to take a broader look at the data and to consider differences between the relative hazard rankings for each chemical group. At this level of analysis the **experiment** becomes the full set of tests on a group of compounds and the **hazard ranking** itself becomes the **output** of an experiment, as outlined by Giddings [9]. These rankings are hypothesized to be more consistent among different ecosystems, and hence

more generalizable, than would qualitative or quantitative predictions of effects.

MATERIALS AND METHODS

Only ecosystem-level state variables were measured. Our justification for treating pH and dissolved oxygen (DO) as measures of the state of the ecosystem (i.e., as ecosystem variables) is based on a paper by Schindler et al. [13]. Figure 1 presents a conceptual model of an ecosystem as an integrated biogeochemical system. In a system without biological activity the geochemical matrix (the nutrient medium in our laboratory ecosystems) can be considered to be in a thermodynamic ground state, or equilibrium, which is completely characterized by its pH and oxidation-reduction (or redox) potential [14]. With the addition of a complex mixture of biota comprising producers, consumers and decomposers (i.e., an "ecosystem seed" or inoculum) and a source of light energy, coupled anabolic and catabolic processes give rise to a non-equilibrium thermodynamic state determined by the ecological energy flow and nutrient cycling processes occurring within the system, which is now an eco-system. This biological activity, through electron and proton transfers, produces changes in the pH and redox potential of the aqueous phase, and the resulting normal range of these values can be considered to represent the unperturbed state of the system. Perturbations to the ecosystem, if they affect energy flow or nutrient cycling processes, will produce corresponding changes in pH and redox potential and move one or both out of the normal range. Recovery occurs when pH and redox potential

return to their normal range. Following Waide, et al. [15], we assumed that DO provided a suitable measure of redox potential in aerobic systems. Therefore we used pH and DO as integrated measures of the processes occurring within the microcosms and of changes in those processes due to toxic chemical effects. In earlier work we found these measures to be reliable indicators of effect which can be more sensitive than species counts [16].

Mixed flask culture microcosms were developed according to the method of Leffler [8,17]. These microcosms consisted of one dm^3 (liter) beakers containing 950 cm^3 (ml) of Leffler's modification of Taub #36 medium [17,18] and 50 cm^3 of a mixed culture inoculum. The inoculum was taken from a mature stock community composed of zooplankton, algae and other microorganisms collected from local lake and pond communities. We were not concerned with species composition other than to verify that the inoculum contained the following functional groups: two species of unicellular green algae, one species of nitrogen fixing blue-green algae, one species of filamentous green algae, one species of herbivorous grazer, one species of benthic detritivore, bacteria and protozoa.

Microcosm containers were not covered, in order to allow free exchange of gases with the atmosphere. Evaporative losses provided sufficient free volume (at least 50 cm^3) for weekly re-inoculation to simulate natural immigration and to provide the potential for system recovery.

The microcosms were arranged in a randomized block design in a Percival I-60 Environmental Growth Chamber at 293 K (20 C). Fluorescent light banks provided illumination on a 12 hour light-dark cycle. To guard

against potential effects from non-uniform incubator conditions, all blocks were rotated to a different shelf position twice weekly.

For each test 40 microcosms were started and followed through a six week pre-dose development phase. Prior to treatment the five microcosms showing the greatest deviation from the group were culled, so that the final treatment design consisted of 35 microcosms divided into control and treatment groups of five replicates each. These systems were then monitored for six weeks following treatment.

Dissolved oxygen was determined electrochemically using an Orbisphere Model 2607 in early experiments and an Altex (Beckman) Model 0260 Oxygen Analyzer in later work. Measurements of pH were taken using a Fisher 525 meter attached to a junction box equipped with standard pH electrodes. Although no counts of population densities were made, qualitative observations of change in algal or zooplankton abundance were recorded.

Post treatment monitoring was done on a schedule of decreasing frequency. Measurements were made on four days the first week, two days the second week and once weekly thereafter. On each sampling day both "pre-dawn" and late afternoon measurements were taken.

The compounds tested were chosen from the extensive set of toxicity data on the fathead minnow (Pimephales promelas) developed for the quantitative structure-activity relationships (QSAR) program at the U.S. EPA Environmental Research Laboratory in Duluth, MN (unpublished data base, ERL-Duluth). Toxicity data on over 450 compounds in over 40 chemical categories provided a wide choice of toxicity levels and modes of action. Three chemical groups (FIG. 2) were chosen to represent distinctly

different examples of possible groups of compounds with different environmental chemistry and modes of action[3].

Treatment levels were chosen on the basis of the fathead minnow LC50 values, microcosm range-finding tests and solubility considerations. In selecting treatment levels, the reported fathead minnow LC50 was used as the median concentration. Other concentrations were set, if solubility limits permitted, two orders of magnitude above and below this level. Acetone was used as a carrier solvent for most solid compounds and sparingly-soluble liquids. Methanol was the solvent for 4-hexyloxyaniline, a solid, because it appeared to degrade in acetone. When a solvent was necessary, a volume not exceeding 10^{-4} v/v was added to all treated systems and to a set of solvent controls, thereby reducing the number of toxicant treatment levels from six to five. Microcosms were dosed once only, so that toxicant levels presumably declined over time and systems had the potential for recovery.

The term "concentration" as used in this paper is not the aqueous phase concentration (except perhaps immediately after the toxicant is added), but is instead the nominal concentration, i.e., the total measured amount of toxicant added per unit volume of the microcosm. When a toxicant is added to a microcosm test system we are dosing the entire ecosystem, not specifically treating the organisms within the microcosm. In this respect microcosm tests are different from single-species or multi-species exposure tests. Once a toxicant is added to a microcosm it is quickly partitioned among the biota, the liquid phase, and other abiotic components of the system. Therefore a measurement of toxicant in the liquid phase, unless taken immediately after addition, is of little value in

determining the amount of toxicant in the system.

Dunnett's procedure [19] was used to determine which treatment groups were significantly different from the control. With this information, lowest observable effect levels (LOEL) were determined individually for each measured variable at each measurement time. The LOEL for the microcosm test as a whole was chosen as the lowest measured variable LOEL during a specified time period, either one to four days (96 hr) or greater than 4 days. A combination of variables, such as diurnal oxygen gain or loss, was generally less sensitive than the individual variables due to what we interpreted as the random addition of errors. In general we have found that the use of the primary variables directly is, at least statistically, a more sensitive measure.

A typical data set for a given variable is illustrated by Figure 3, in which the difference between the mean of a measured variable and the control group mean is plotted versus time. Dunnett's least significant difference [19] for the group is shown as a shaded area. This type of figure clearly shows the relationship between toxicant concentration and ecosystem dysfunction and recovery. Since we were making four separate statistical comparisons (morning and evening measurements for DO and pH) on each of ten measurement days (for a total of 40 tests), we would expect that two of these tests (5%) might show a significant difference just by chance at $p = 0.05$. To reduce this possibility, we required that statistically significant differences from controls occur on at least two <u>consecutive</u> sampling times (consecutive mornings or consecutive evenings) before we considered an effect to be real.

Results

Since the compounds tested were selected from a structure-activity data base on the fathead minnow to represent different chemical groups and modes of action, the fathead minnow is the only species on which all of our test compounds have been evaluated. Accordingly, 96 hr LC50 data from these tests were used to establish a rank order against which relative toxicity to microcosm communities could be compared. The data from the three intragroup comparisons are summarized in Table 1. Within each group, the compounds are listed by their abbreviated names in the order of their relative toxicity to fathead minnows. The fathead minnow 96hr LOEL, rather than the LC50, is listed to provide a more comparable measure of the relative sensitivity of the microcosm test compared to this single species test. The last two columns give the lowest observed ($p \leq 0.05$) effect levels (LOEL) for the microcosms determined from morning or evening DO or pH measurements. LOEL's are listed separately for effects occurring during the first 96 hours and for effects occurring between four and 42 days after treatment. The alcohols and tetrachloroaniline were tested twice, as previously reported [11,12]. Where LOEL's differed between tests the lowest effect level is the one recorded here. The other compounds were tested once.

The relationships between hazard rank order and relative sensitivity within these three chemical groups are summarized in FIG. 4. Here the LOEL's listed in Table 1 are plotted on an identical log (base 10) scale for each group so that the relative toxicities of chemicals in each group can be easily seen by noting the position of an LOEL on the ordinate. Toxicity

differences between pairs of adjacent compounds and between most and least toxic members of a group can be compared by noting the slope of the appropriate lines.

In the alcohol group, the short-term ecosystem LOEL's for decanol and octanol were close to the fathead minnow 96hr LOEL values in both absolute value and rank order. Hexanol however had a 96hr ecosystem LOEL of 97.9 mol/m^3, considerably below the fathead minnow LOEL of 863 mol/m^3. Therefore, although the hazard ranking is similar for the microcosm and fathead minnow tests, the microcosm test was more sensitive for hexanol, the least toxic member of the series. The long-term LOEL's for the alcohols are equal to or higher than the short-term LOEL's, since the lower treatment-level groups often recovered to control levels within four days of treatment.

With the exception of tetrachloroaniline, the aniline group of compounds were more toxic to microcosms than to fathead minnows. The magnitude of this difference was especially large for diisopropylaniline and aniline. Based on long term microcosm LOEL's one could predict that there is unlikely to be any significant difference in long-term toxicity between hexyloxyaniline, diisopropylaniline and aniline in natural ecosystems.

The ecosystem responses to the substituted aromatic amides differed from the fathead minnow LOEL's both in sensitivity and in rank order. Among the three compounds the microcosms were most sensitive in 96 hr to diuron, whereas fathead minnows were least sensitive. Short-term ecosystem effects were evident at greater than two orders of magnitude below the fathead 96hr LOEL. Similarly, octyloxyacetanilide produced

short-term ecosystem effects at a 1/10 the fathead 96hr LOEL. The microcosms were less sensitive to salicylanilide than the fathead minnow during the first 96 hours. With the exception of salicylanilide, long-term effects appeared in the microcosms in this chemical group at 1/10 of the short-term effect levels.

Discussion

For general depressants or narcotics, which have similar effects on most or all organisms, we expected that ecosystem-level measures of effect would rank toxicities in the same order as single-species toxicity tests. Other compounds, belonging to groups with specific or even multiple modes of action or having complex degradation pathways with potential toxic intermediates, would not necessarily exhibit a high correlation between ecosystem-level effects and any particular single species toxicity test. This is because of the greater opportunity for complex interactions to occur in ecosystems between differently-affected species in groups for which different modes of action are effective.

To paraphrase Giddings [9], it is the ranking of chemicals in order of the concentrations required to produce an observable effect, not the observed effects themselves, that constitutes the output of this experimental design. Our model ecosystems were used to identify chemicals capable of disrupting ecosystem processes, not to specify which processes are disrupted or precisely how these effects might be manifested in natural systems. Since many single-species toxicity tests also have the objective of ranking chemicals by potential hazard, model

ecosystems would be valuable primarily if they were more sensitive than conventional single-species tests or if they generated different rankings than those of conventional tests. If ecosystem-level tests merely echoed the results of simpler, more easily standardized tests, their use for screening chemicals would be questionable. Our results suggest that this relatively simple generic laboratory ecosystem test is promising on both counts.

Straight-chain alcohols represent general narcotics, acting to depress biological activity over all or most organisms. Ecosystem relative toxicity of three alcohols not only followed the single species rankings but, with the exception of hexanol, were well within an order of magnitude of the fathead minnow 96hr LOELs.

Aniline and derivatives act not only as general narcotics but in other ways, and also have a more complex environmental chemistry with toxic degradation intermediates [20]. This was reflected in microcosm responses to these compounds, where four day LOELs indicated relative toxicity similar to that seen in fathead minnow tests. Long-term effects produced a different ranking, however, possibly in response to degradation products.

Many compounds in the aromatic amide group affect photosynthesis and thus would be expected to have a dramatic effect on ecosystems. Salicylanilide contains a phenolic group which causes it to primarily act as an uncoupler of oxidative phosphorylation [21]. Classification of these compounds by means of the amide functional group obviously has little relevance to the mode of action when the compounds contains other functional groups which are more important toxicologically. One would

expect, therefore, that an ecosystem-level hazard ranking of chemicals in this group might be considerably different than a hazard ranking based on any particular single species test. Diuron, the amide least toxic to fathead minnows, is a potent inhibitor of photosynthesis and was in fact the most toxic of all the compounds tested in microcosms. Octyloxyacetanilide, whose mode of action is not known to us at present, was nearly as toxic at the ecosystem level as Diuron, although differing from that compound in toxicity to fathead minnows by nearly two orders of magnitude.

These examples can be considered representative of situations that might be encountered in an early stage of ecosystem-level testing, and illustrate how, for less well known toxicants, microcosm functional testing could assist in identifying chemicals which require more elaborate test procedures. In such cases ecosystem-level testing of a new compound or several members of a class of compounds, perhaps in conjunction with an appropriate reference chemical or benchmark, can provide a trigger for initiating additional testing. We believe that these results support simultaneous toxicity testing at different levels of biological organization [7]. For example, if a chemical manufacturer is developing a new product in a chemical structure group about which little knowledge exists for predicting toxic mode of action, a relatively simple ecosystem-level test including one or more benchmark chemicals could indicate whether additional tests are desirable and also what kind of additional tests might be needed. Although it is not an essential part of this test protocol, qualitative or semi-quantitative observations of the early effects of toxicants on component trophic groups in the microcosms

can also point toward additional tests on certain taxa.

Provided that measurements are restricted to characteristics of the state of the whole system, measuring ecosystem-level effects in the laboratory is not inherently more difficult than measuring single species effects. The six-week development period need not be considered as part of the test, and is analogous to the acclimation period for organisms brought in from the field or to the time required to produce the desired life stage for testing. At the most intense level of testing we had three tests underway simultaneously; one in the pre-dose phase, one in the two-week post-dose period where measurements were made most frequently, and one in the last four weeks of observation where measurements were made only once per week.

More relevent, however, is the effort required to obtain ecosystem-level hazard rankings of chemicals by other means. In this regard laboratory ecosystems are definitely less labor intensive than tests in outdoor ecosystems, which in many cases are not even feasible to perform.

Although we consider these results to provide support for ecosystem-level testing, we do not yet consider the results to be conclusive. There is a need for additional testing with different groups of compounds, for additional replication of tests on the same compounds, and for studies of the influence of aspects of the test protocol such as media formulation and microcosm development time.

Acknowledgement - We gratefully acknowledge the participation of Carl Mach, Terry Flum, Charles Walbridge, and Daniel Fitzsimmons in the collection of data and other essential laboratory activities. Dr. Michael Harrass worked with our group during the first year of the project. Dr. Jack Hargis, of the University of Minnesota, Duluth, participated in the initial conception of the research but did not live to see the fruits of his efforts. Laboratory space, supplies and equipment were largely supplied by the Environmental Protection Agency, Environmental Research Laboratory - Duluth (MN), and support of non-EPA personnel was provided through EPA cooperative agreement #CR810741

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TABLE 1--Comparison of ecosystem vs. fathead minnow hazard rankings for selected toxicants.

Chemical	Fathead 96hr LOEL (mol/m^3)	Microcosm 96hr LOEL (mol/m^3)	Microcosm >96hr LOEL (mol/m^3)
Straight-chain Alcohols			
Decanol	11.	19.	19.
Octanol	68.	77.	77.
Hexanol	863.	97.9	979.
Anilines			
Tetrachloroaniline	0.87	1.2	1.2
Hexyloxyaniline	14	6.7	14.
Diisopropylaniline	59.8	8.5	8.5
Aniline	851.	107.	10.7
Aromatic amides			
Octyloxyacetanilide	<1.2	0.19	0.019
Salicylanilide	8.25	46.2	46.2
Diuron (DCMU)	24.	0.12	0.012

Chemicals are listed within a group in order of fathead minnow toxicity. Numbers are Lowest Observed Effect Level (LOEL) for both fathead minnow and microcosm. Effect is lethality for fatheads, pH or DO change for microcosms. Full names of chemicals are given in footnote 3.

Footnotes

1. U.S. Environmental Protection Agency, Environmental Research Laboratory, Duluth, MN 55804

2. Department of Biology, University of Minnesota, Duluth, MN 55812

3. 1-decanol (CAS#112-30-1); 1-octanol (CAS#111-87-5); 1-hexanol (CAS#111-27-3); 2,3,5,6-tetrachloroaniline (CAS#3481-20-7); 4-hexyloxyaniline (CAS#39905-57-2); 2,6-diisopropylaniline (CAS#24544-04-5); aniline (CAS#62-53-3); 2-(octyloxy)acetanilide (CAS#none); salicylanilide (CAS#87-17-2); diuron, or DCMU, or 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (CAS#33-05-4)

Figure Captions

FIG. 1--Conceptual model of an ecosystem as an integrated biogeochemical system. Adapted from [13].

FIG. 2--Skeletal structures and molecular weights of test compounds.

FIG. 3--Typical data set, in which the deviation of a variable (DO) is plotted versus test day. Shaded area is the region of non-significant difference, from Dunnett's test [19]. Toxicant is added on day 42, and measurements normally continue through day 84. DO deviations in mol/m^3 = 31.25 x mg/L.

FIG. 4--Comparison of ecosystem vs fathead minnow hazard rankings for selected toxicants. LOEL is Lowest Observed Effect Level, in mol/m^3. Effect is lethality for fatheads, pH or DO change for microcosms. µcosm is microcosm. Full names of chemicals are in footnote 3.

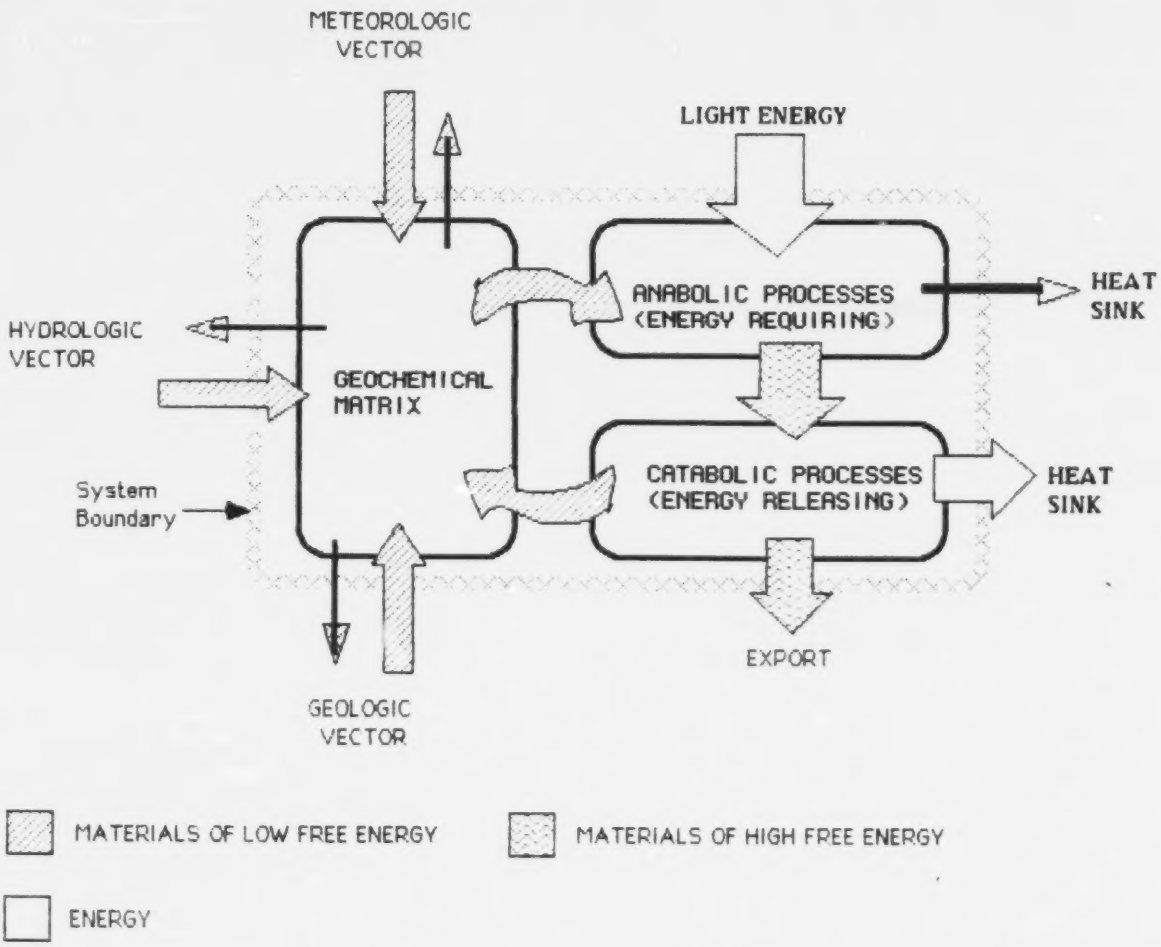

METEOROLOGIC
VECTOR
LIGHT ENERGY
HYDROLOGIC
VECTOR
GEOCHEMICAL
MATRIX
ANABOLIC PROCESSES
(ENERGY REQUIRING)
HEAT
SINK
CATABOLIC PROCESSES
(ENERGY RELEASING)
HEAT
SINK
System
Boundary
EXPORT
GEOLOGIC
VECTOR
MATERIALS OF LOW FREE ENERGY
MATERIALS OF HIGH FREE ENERGY
ENERGY

FIGURE 1

Yount, J. David and Lyle J. Shannon

Functional responses of laboratory microecosystems to chemicals from three structural groups.

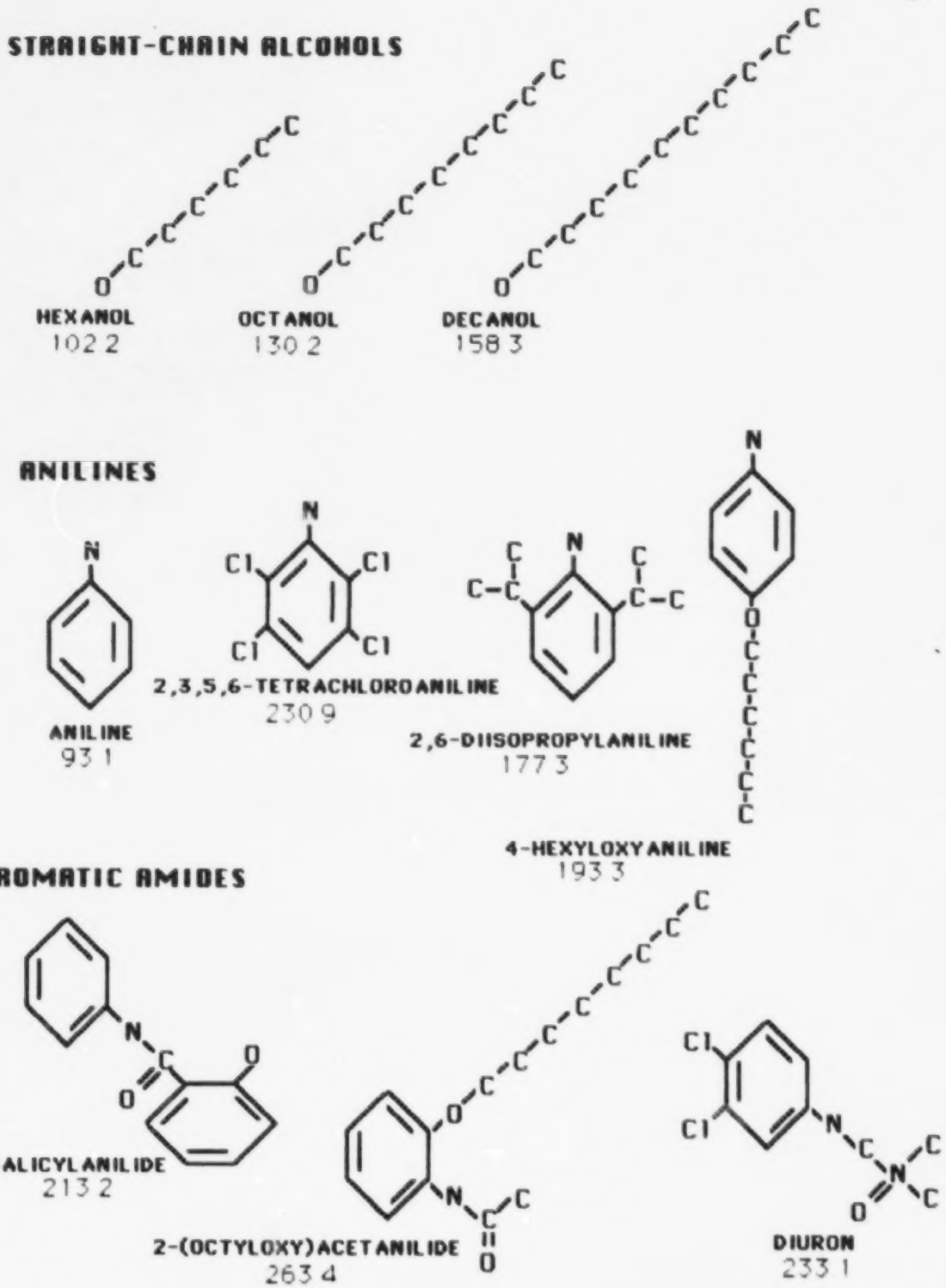

STRAIGHT-CHAIN ALCOHOLS
HEXANOL
102 2
OCTANOL
130 2
DECANOL
158 3
ANILINES
ANILINE
93 1
2,3,5,6-TETRACHLOROANILINE
230 9
2,6-DIISOPROPYLANILINE
177 3
4-HEXYLOXYANILINE
193 3
AROMATIC AMIDES
SALICYLANILIDE
213 2
2-(OCTYLOXY)ACETANILIDE
263 4
DIURON
233 1

FIGURE 2

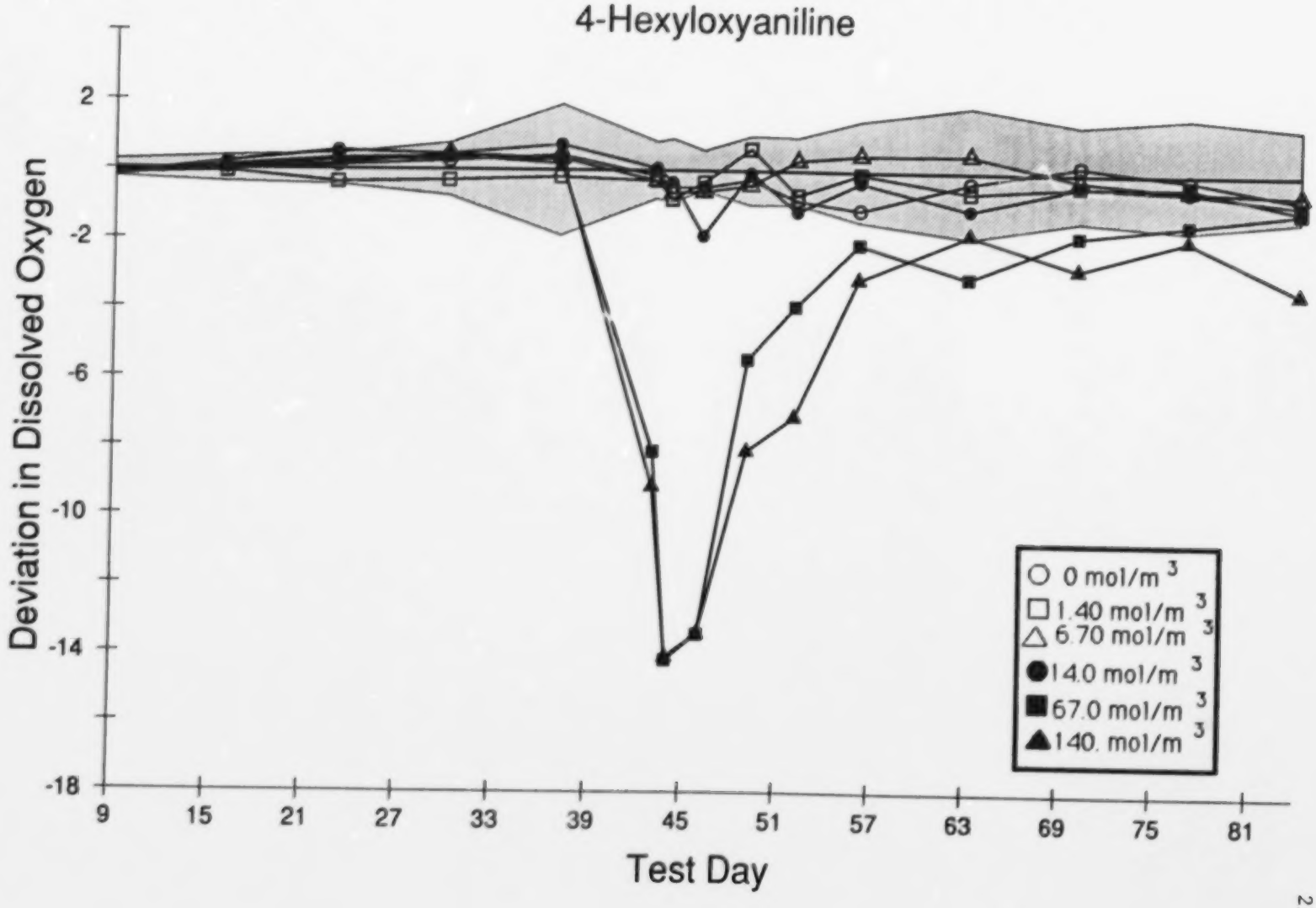
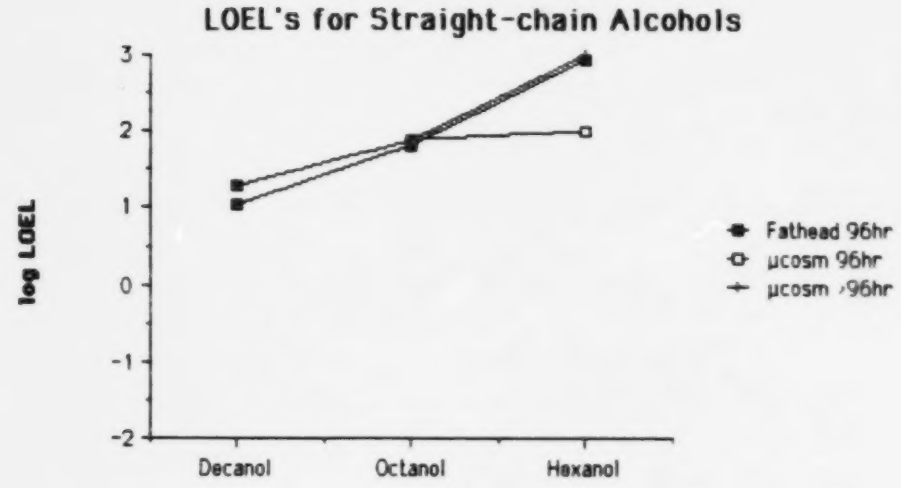
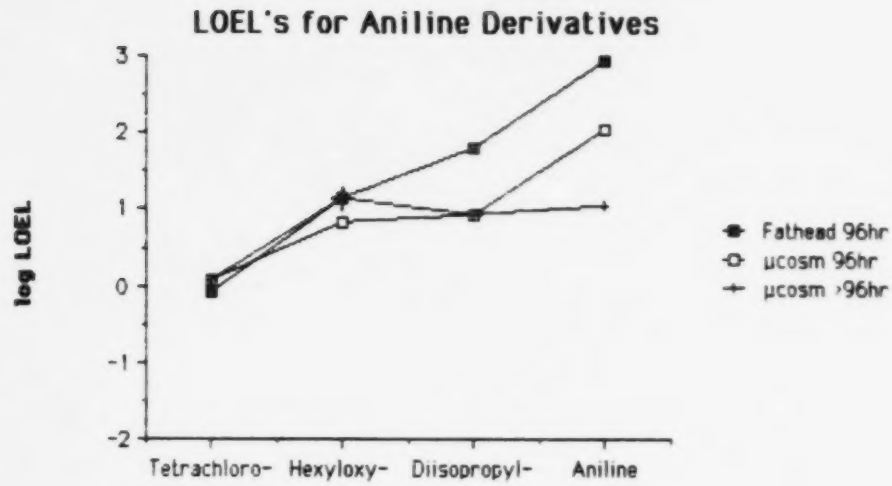

4-Hexyloxyaniline
Deviation in Dissolved Oxygen
2
-2
-6
-10
-14
-18
9
15
21
27
33
39
45
51
57
63
69
75
81
Test Day
0 mol/m 3
1.40 mol/m 3
6.70 mol/m 3
14.0 mol/m 3
67.0 mol/m 3
140. mol/m 3

FIGURE 3


LOEL's for Straight-chain Alcohols
log LOEL
3
2
1
0
-1
-2
Decanol
Octanol
Hexanol
Fathead 96hr
µcosm 96hr
µcosm >96hr


LOEL's for Aniline Derivatives
log LOEL
3
2
1
0
-1
-2
Tetrachloro-
Hexyloxy-
Diisopropyl-
Aniline
Fathead 96hr
µcosm 96hr
µcosm >96hr

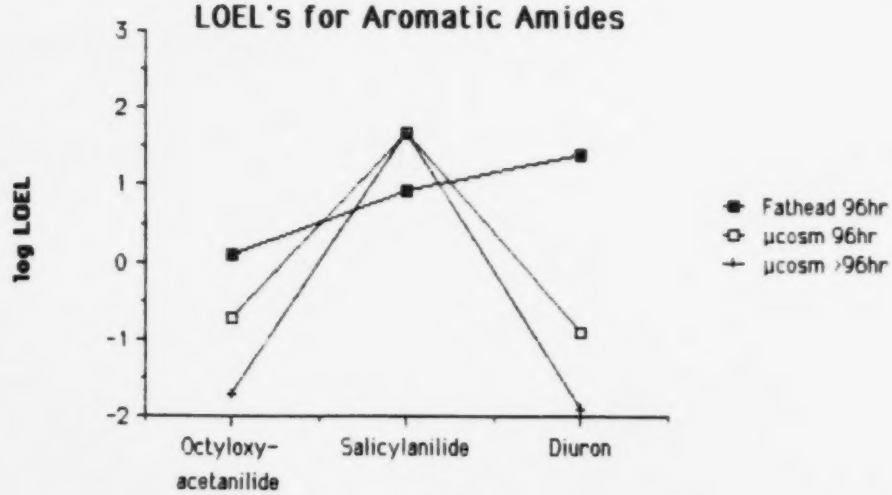

LOEL's for Aromatic Amides
log LOEL
3
2
1
0
-1
-2
Octyloxy-
acetanilide
Salicylanilide
Diuron
Fathead 96hr
µcosm 96hr
µcosm >96hr

FIGURE 4